Pantothenate biosynthesis in higher plants


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Abstract
Pantothenate (vitamin B₅) is a water-soluble vitamin essential for the synthesis of CoA and ACP (acyl-carrier protein, cofactors in energy yielding reactions including carbohydrate metabolism and fatty acid synthesis. Pantothenate is synthesized de novo by plants and micro-organisms; however, animals obtain the vitamin through their diet. Utilizing our knowledge of the pathway in Escherichia coli, we have discovered and cloned genes encoding the first and last enzymes of the pathway from Arabidopsis, panB1, panB2 and panC. It is unlikely that there is a homologue of the E. coli panD gene, therefore plants must make β-alanine by an alternative route. Possible candidates for the remaining gene, panE, are being investigated. GFP (green fluorescent protein) fusions of the three identified plant enzymes have been generated and the subcellular localization of the enzymes studied. Work is now being performed to elucidate expression patterns of the transcripts and characterize the proteins encoded by these genes.

Introduction
Pantothenate or pantothenic acid is a water-soluble vitamin more commonly referred to as vitamin B₅. It is synthesized by micro-organisms and plants; however, it is necessary for animals to obtain it through their diet [1]. The word pantothenate is derived from the Greek word panto(s), meaning everywhere, due to it being found in most foodstuffs [2]. This fact is supported by a lack of pantothenate deficiencies reported in nature. Pantothenate is the precursor of the 4′-phosphopantetheine moiety of CoA and ACP (acyl-carrier protein) [3]. CoA and ACP are found in all cell types, with CoA playing an essential role in central metabolism such as the tricarboxylic acid cycle, fatty acid oxidation and isoprenoid biosynthesis, and ACP involved in the synthesis of lipids. In plants CoA is also important in many aspects of secondary metabolism, including lignin biosynthesis.

From two aspects, the pantothenate pathway is of great commercial interest. Firstly, the synthesis of pantothenate for addition to cosmetics, such as hand cream and shampoo, or to be sold as a vitamin supplement, is carried out on a large scale, yielding approx. 5000 tons/year [4]. However, this can be an expensive process due to the necessity of separating the biologically active D-pantothenate from the L-form in the racemic mixture. Overexpression of pantothenate in plants and micro-organisms would not require this purification step and so, after optimization, may result in more economical production of the vitamin. Secondly, as pantothenate biosynthesis occurs in plants and micro-organisms and not in animals, the enzymes of the pathway are ideal targets of potential herbicides, fungicides and antibiotics.

The pantothenate pathway was first elucidated in Escherichia coli where it is best understood, and known to consist of four enzymatic steps in two separate branches (see [26]). The initial step of the pathway involves the transfer of a hydroxymethyl group from 5,10-methylene tetrahydrofolate to α-KIVA (α-ketoisovalerate) generating ketopantoate, catalysed by KPHMT (ketopantoate hydroxymethyltransferase). Ketopantoate is reduced to pantoate by KPR (ketopantoate reductase) [5] in an NADPH-dependent reaction. β-Alanine is formed by decarboxylation of l-aspartate catalysed by l-aspartate-α-decarboxylase in a separate branch of the pathway [6]. Formation of pantothenate in the final step of the pathway occurs through an ATP-dependent condensation reaction between pantoate and β-alanine carried out by PS (pantothenate synthetase) [7].

Biochemical studies of the pantothenate pathway in plants
The first indication of the presence of the pantothenate pathway in plants came from the identification of a pantothenate-requiring auxotroph of Datura innoxia (thorn apple) [8,9]. Growth of the mutant tissue culture cells was supported by addition of ketopantoate, pantoate and pantothenate to the growth medium. However, addition of α-KIVA had no effect indicating a deficiency in the capacity to convert α-KIVA to ketopantoate, in other words absent or defective KPHMT. In order to determine whether functional KPHMT was present in the mutant line, Sahi et al. [9] attempted to assay the activity of the enzyme, but the activity of KPHMT could not be reliably detected in either the auxotroph or the wild-type.

Further evidence to indicate the presence of the pantothenate pathway in plants, similar to that in bacteria, came from feeding studies with radiolabelled L-[14C]valine [10]. Pea (Pisum sativum) leaf discs were fed with radiolabelled valine from which α-KIVA is derived by transamination.

Key words: Arabidopsis, higher plants, ketopantoate hydroxymethyltransferase (KPHMT), pantothenate biosynthesis, pantothenate synthetase, subcellular localization.

Abbreviations used: ACP, acyl-carrier protein; ADC, aspartate decarboxylase; GFP, green fluorescent protein; α-KIVA, α-ketoisovalerate; KPHMT, ketopantoate hydroxymethyltransferase; KPR, ketopantoate reductase; PS, pantothenate synthetase.

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HPLC analysis of the different compounds eluted showed radiolabelled fractions corresponding to α-KIVA, keto-pantooyl lactone (derived from ketopantoate) and pantoyl lactone (derived from either pantoate or pantotenate). The formation of radiolabelled intermediates along each step of the pathway known in *E. coli* suggested the pathway to be similar in plants, and provided support for the existence of the enzymes KPHMT and KPR.

Identification of genes encoding pantothenate biosynthesis enzymes in plants

Cloning of the genes for pantothenate biosynthesis enzymes in *E. coli* has resulted in both sequence information and the crystal structures of the proteins, which have subsequently provided valuable tools to search for homologous genes in higher plants. The identification of these genes has afforded further insight into the operation of the plant pathway.

**KPHMT**

KPHMT, the first enzyme of the pathway, is encoded by *panB*. The *E. coli* *panB* gene was used as a query in a BLAST search of the *Arabidopsis thaliana* genome. This resulted in the identification of two *panB* genes, *panB1* and *panB2*, with 87% similarity to each other at the amino acid level [11]. The occurrence of two *panB* genes in the *Arabidopsis* genome is likely to be the result of genome duplication, since the two genes lie in regions of chromosomes II and III where a duplication event has taken place [12]. The two *panB* genes were amplified from an *Arabidopsis* cDNA library, and found to functionally complement the *E. coli* *panB* mutant Hfr3000 YA139, indicating that both genes encode active proteins [11]. Furthermore, two *panB* genes are found in the completed rice genome, suggesting that the presence of two KPHMT isoforms might have functional significance in plants.

**PS**

The final enzyme of the pathway, PS catalyses the ATP-dependent condensation of pantoate and β-alanine, generating pantotenate. The *E. coli* *panC* mutant was used in an attempt to identify the *panC* gene in a number of plant cDNA libraries. Functional complementation of the auxotroph proved successful when screening the *Lotus japonicus* cDNA library [13]. *Lotus panC* encoded a peptide sequence of 308 amino acids. An EST (expressed sequence tag) from rice (*Oryza sativum*), showing similarity to the *E. coli* *panC*, encoded a 313 amino acid peptide. Neither of the plant enzymes had an N-terminal extension compared with the bacterial enzyme, but there was a 20–30 amino acid insertion in the middle, which corresponds to a region near the dimer interface of the *E. coli* PS structure [14]. Overexpression and purification of recombinant *Lotus* PS allowed kinetic characterization of the enzyme [15]. Normal saturation kinetics was observed for β-alanine, whereas pantoate concentrations above 0.5 mM showed inhibition of the activity of the enzyme; the enzyme from *E. coli* has normal saturation kinetics for both substrates. In contrast, the *Lotus* enzyme is not inhibited by its product [15].

After the release of the genome sequence by the *Arabidopsis* Genome Initiative, the *panC* gene was identified and was cloned from an *Arabidopsis* cDNA library. It was established to encode a functional protein by complementation of the *E. coli* *panC* mutant.

**β-Alanine synthesis**

In *E. coli*, β-alanine is synthesized from l-aspartate via a decarboxylation reaction catalysed by ADC (aspartate decarboxylase); however, no homologues of ADC were found in the *Arabidopsis* genome by BLAST searching. Furthermore, functional complementation of the *E. coli* *panD* mutant DM 3498 using an *Arabidopsis* cDNA expression library resulted in no positive clones. An alternative method was used to determine whether ADC is encoded in the *Arabidopsis* genome. FUGUE is a novel search approach, which compares the query sequence to a database of proteins with solved crystal structures [15]. Reverse-FUGUE can also be performed whereby a solved crystal structure can be used as the query to search a specific predicted proteome. The structures of all the *E. coli* pantothenate biosynthesis enzymes were used in a reverse-FUGUE search of the annotated *Arabidopsis* proteome. Despite identifying the two genes for KPHMT and the single gene for PS, no convincing hit for ADC was obtained. Furthermore, no ADC homologue was identified in *Saccharomyces cerevisiae* by this approach, indicating that the gene may not have traversed the prokaryotic–eukaryotic border [11]. Instead, like other eukaryotes, it is likely that plants have alternative methods by which β-alanine is produced. In yeast, β-alanine for pantothenate biosynthesis appears to come from the polyamine spermine [16]. The degradation of uracil and thymine to produce CO₂, NH₃, β-alanine and γ-aminoisobutyrate has been shown to occur in seedlings of oil seed rape [17] and a gene for one of the enzymes in maize has been characterized [18]. An alternative method of β-alanine production, from 3-oxopropanoate, may be involved in pantothenate production, after the identification of β-alanine aminotransferase in *Arabidopsis* [19]. It is still unclear if any of these methods play a role in the production of β-alanine specifically for use in the pantothenate pathway. Furthermore *Arabidopsis* can survive without the ability to make spermine [20]. A more detailed explanation of sources of β-alanine in plants can be found in the review by Raman and Rathinasabapathy [21].

**KPR**

Owing to the identification of the first and final enzymes of the pantothenate pathway in the *Arabidopsis* proteome, it is inferred that the intermediate enzyme KPR is also present. Attempts were made to identify KPR by BLAST searches of the *Arabidopsis* genome, using the *E. coli* *panE* sequence as the query, but no homologue was found. Since sequence similarity in *panE* between micro-organisms is poor, failure to find a homologue of a plant sequence using a...
Comparison of the enzymes of the pantothenate biosynthetic pathway from *E. coli*, *Arabidopsis* and *S. cerevisiae*

This figure shows the schematic representation of the alignment of the mature regions of the enzymes involved in pantothenate biosynthesis, with the shaded areas showing extensions relative to the *E. coli* enzyme. The extensions of the *Arabidopsis* and *S. cerevisiae* KPHMT at the N-termini have been shown to target the enzymes to the mitochondria [11,24]. The C-termini extensions are of unknown function. The two eukaryotic PS are known to localize to the cytosol despite the N-terminal extension seen in the *S. cerevisiae* enzyme. *S. cerevisiae* KPR, showing no extension relative to *E. coli*, is found in the cytosol [24]. An *Arabidopsis* KPR has not yet been identified; however by inference, it is likely to be found in the cytosol.

**Figure 1**

Subcellular organization of the pantothenate pathway in plants

Elucidation of the pantothenate pathway in *Arabidopsis* has raised many questions. For example, why are there two functional copies of KPHMT in plant proteomes? It is possible that the proteins are functionally redundant, or it may be that the two isoforms are localized to discrete parts of the cell. It is possible that the genes are differentially expressed. Sequence alignment of KPHMTs from prokaryotes and eukaryotes clearly shows that the eukaryotic proteins have N-terminal and C-terminal extensions compared with the prokaryotic ones (Figure 1), with the possibility that these are involved in subcellular targeting. To answer this question, cDNAs encoding C-terminal fusions of GFP (green fluorescent protein) to the pantothenate biosynthetic enzymes were generated. These constructs were used to transform onion epidermal cells and tobacco epidermal and guard cells by biolistic transformation. Confocal microscopy showed both isoforms of KPHMT targeted GFP to the mitochondria, confirmed by co-localization with formate dehydrogenase (FDH), an enzyme known to target to the mitochondria of plants, fused to red fluorescent protein. Thus both KPHMT1 and KPHMT2 are targeted to the mitochondria (Figure 2). The discovery that KPHMT is targeted to the mitochondria led to a breakthrough in the ability to measure the activity of the enzyme after previous attempts had proved unsuccessful [13,9]. Purified mitochondria from pea leaves and *Arabidopsis* suspension cultures were found to have measurable activity, whereas none was detected in isolated chloroplasts or boiled mitochondria [11]. In contrast with the mitochondrial location of KPHMT, PS-GFP was shown to be cytosolic (Figure 2). This spatial organization of the enzymes implies that either ketopantoate or pantoate must pass out of the mitochondria into the cytosol. Recent work performed on the subcellular localization of the entire *S. cerevisiae* proteome has identified both KPR and PS as cytosolic enzymes, whereas KPHMT is in the mitochondrial [24]. By inference, the *Arabidopsis* KPR homologue may well be cytosolic and thus it is anticipated that ketopantoate traverses the plant mitochondrial membrane.

It can be concluded that differential subcellular localization is not the rationale behind the *Arabidopsis* proteome containing two functional KPHMTs. It is possible that the two KPHMT isozymes are differentially expressed. Studies are being carried out using promoter::GUS fusions, Western blot analysis and RT (reverse transcriptase)–PCR to determine the patterns and levels of expression of the enzymes in higher plants.

**Conclusions**

Plants have been shown to possess two functional copies of the gene, *panB*, encoding the first enzyme and one copy of *panC*, encoding the final enzyme of pantothenate biosynthesis. Possible homologues to KPR have been identified using structure similarity searchers, but further investigation is required to determine whether any of these proteins have KPR activity. Nevertheless, there will be an enzyme that catalyses this second step of the pathway. In contrast, plants are unlikely to make β-alanine using ADC. Targeting studies
Figure 2 | Subcellular localization of the Arabidopsis pantothenate biosynthesis enzymes and organization of the pathway in plant cells

Particle bombardment was used to introduce constructs carrying panB1-GFP, panB2-GFP and panC-GFP fusions into onion epidermal cells. After incubation in the dark for 16–24 h at 25°C on 1/2 MS agar, the transformed cells were visualized by confocal microscopy. The GFP fluorophore was excited using an argon ion laser line of 488 nm and the emission spectra of the excited GFP were collected between 520 and 580 nm.

using GFP fusions indicated localization of both KPHMT isoforms to the mitochondria, whereas PS is in the cytosol, suggesting the need for transportation of either ketopantoate or pantoate across the mitochondrial membrane.

Once the elucidation of the pathway is complete it may enable the exploration of its connections to CoA biosynthesis, for which the pathway has been fully elucidated in plants [25]. In the meantime, we are well situated to start exploring regulation of the pathway both at the level of gene expression and fine regulation of enzyme activity. These data will be invaluable in attempts to overproduce pantothenate in plants.

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References


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